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COMPLETE SPECIFICATION

"Milk Processing and Handling"

We, **DEC INTERNATIONAL NZ LIMITED**, a duly incorporated New Zealand company of 558 Te Rapa Road, Hamilton, New Zealand, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:

The invention relates to milk processing.

At present milk, after it is taken during milking from a cow or other animal, is transported in bulk to a milk treatment station. At the milk treatment station the milk in bulk is processed in a number of ways to produce either milk, powdered milk, cheese or other products or milk derivatives.

With cheese production there is available as a by-product a large volume of whey. With butter production butter milk occurs as the major aqueous by-product of processing. In these cases, the whey or butter milk which has a relatively large volume is then either further processed at the milk treatment station, is processed as a waste effluent, or is shipped elsewhere for further treatment such as fractionation to remove other useful constituents of milk such as vitamins, carbohydrates, minerals, antibodies, proteins, peptides, amino acids and other useful biomedical or biochemical products which are components of milk. Generically this type of processing is often called bioprocessing which covers all the unit processing steps which maybe found in the extraction of a biochemical from biological feed stock.

A disadvantage of standard milk production processes are the high transportation costs in shipping large volumes of milk or milk by product streams and the delay between milking and removal of useful biomedical or biochemical products.

An object of the present invention is therefore to provide a process and apparatus by which specific constituents of milk, which have biochemical and biomedical interest, can be removed.

An object is also to provide a process or means (including apparatus) useful in isolating endogenous milk proteins such as lacto peroxidase (Lp) and lactoferrin (Lf).

Further objects and advantages of the invention will become apparent from the following description.

According to a first aspect of the invention there is provided a process whereby specific targeted biomedical or biochemical constituents of milk are removed during farm milk handling, the process including:

siting at a farm milking plant means for removing a targeted milk constituent or constituents;

passing milk past or through the means; and

collecting the targeted milk constituent(s) for analysis or further processing.

According to a second aspect of the invention there is provided an apparatus for removing targeted biomedical or biochemical milk constituents, the apparatus being adapted for placement in association with a farm milk plant so that milk, after removal from an animal passes, through the apparatus to remove the desired constituents.

The apparatus may be a modular cartridge type unit which incorporates means for removing specific milk constituents.

The means for removing the milk constituents may be a molecular filtering device or affinity chromatographic means or a combination thereof. For example the molecular filtering device may use ultrafiltration, a chromatographic resin, an ion exchange resin, a molecular sieve resin, an antibody-linked resin, a metal affinity resin or a chromatographic or other impregnated paper or other filter medium. Alternatively the means for removing the milk constituents may use hydrophobic interaction.

The apparatus may be adapted for mounting relative to a milk claw, main milk line or long milk line of the farm milk plant or system.

In yet a further aspect the invention consists in **a method of isolating one or both of the endogenous milk proteins lactoperoxidase** (Lp) and lactoferrin (Lf), said method comprising

presenting the whole milk of one or several animals on or adjacent the milking site of such a mammal or mammals to a matrix with an affinity for the desired endogenous protein to allow the attachment thereof to such matrix, and

thereafter eluting or otherwise removing the desired endogenous protein or proteins from such matrix.

In still a further aspect the invention comprises **a method of isolating an endogenous milk protein from a milk source** which comprises

(i) substantially at the body temperature of the mammal from which the milk is being sourced presenting the whole milk to a matrix with an affinity for the desired endogenous milk protein, and

(ii) thereafter eluting or otherwise removing the desired endogenous protein from the matrix.

Preferably the presenting is at a temperature at which the milk fat remains liquid.

Preferably said isolation is of both endogenous proteins and a selective eluting is used to isolate each protein from the other.

Preferably the matrix is a packed bed column and the matrix is an appropriate matrix for packed bed column chromatography.

Preferably said packed bed column employs an acrylic based support.

Preferably said acrylic based support is BR70 as hereinafter defined.

Preferably the whole milk is presented to the matrix at a temperature within the range of 10°C of the mammals blood temperature and most preferably from 35 to 37°C.

Preferably said eluting is carried out at a temperature within the range of from 35 to 37°C.

Preferably wherein each individual milk feed from a single mammal is presented to a dedicated matrix dedicated to either that mammal or a series of mammals being milked at that station.

Preferably said selective eluting is with a sodium chloride solution.

Preferably Lp is eluted with about 0.25M sodium chloride solution.

In other forms (or preferably as well) Lf is eluted with about a 0.8M NaCl solution.

As used herein the term "biomedical or biochemical constituents of milk" or "endogenous protein(s) of milk" includes:

- beta-lactoglobulin
- alpha-lactalbumin
- serum albumin
- immunoglobulins
- lactoferrin
- lactoperoxidase
- gamma casein
- proteose peptone
- glycoproteins
- beta-casein
- alpha-casein (and the two sub-classes of alpha-S1-casein and alpha-S2-casein)
- kappa-casein
- whey acidic protein
- beta-macroglobulin
- trypsin inhibitor protein
- protease
- growth hormone

If a "transgenic animal" the term may also extend to

- clotting factor IX
- alpha-1 antitrypsin
- tissue plasminogen activator
- follicle simulating hormone
- growth hormone
- interleukin-2

As used herein the term "substantially at the body temperature of the mammal" means preferably within 10°C thereof (preferably just below the temperature - the drop preferably being purely the result to temperature loss during the ducting of milk from the animal to the collection zone (eg. Preferably a cartridge).

In any event for a targeted protein it means preferably a temperature not likely to degrade the protein significantly and a temperature at which milk fat remains liquid.

In another aspect the invention comprises protein isolated by a method of the present invention.

Further aspects of the invention will become apparent from the following description which is given by way of example.

Preferred forms of the present invention will now be described with reference to the drawings in which

Figure 1 appended hereto (the drawing of New Zealand Patent Application No. 280724) shows a diagrammatic layout of a system for employment in accordance with the present invention, and

accompanying Figures 2 through 18 are drawings as hereinafter described in relation to examples including experimentation in relation to the process and the apparatus.

An example of the invention will now be described with reference to the accompanying schematic drawing (Figure 1) which shows a typical example of farm milk plant to which the invention can be attached.

The standard farm milk plant, system or machine has a plurality of pulsator units 1 each of which is connected to a milking claw 2. The output pipes 3 from the milking claws 2 are linked to a milk line 4 at one end of which is a receiver tank 5. Output of air is via pipe 6 from the receiver tank through a sanitary trap (not shown) while milk is drawn (arrow 7) from line 8 to a milk pump (not shown) which feeds the milk to a main holding tank (not shown).

The present invention requires the standard farm milk plant, as outlined above by way of example, to be modified to include a processing step by which targeted milk constituents are removed on site. The means by which the targeted constituents are removed may be a modular cartridge unit (not shown) through which milk passes after

removal from the animal and before it reaches the milk tank.

The modular cartridge unit or units may include any selected filter medium such as a chemical impregnated filter, a chromatographic resin or other molecular filtering device chosen to remove a targeted constituent. The constituent may be any biochemical or biomedical products such as carbohydrates, antibodies, proteins, peptides, minerals or vitamins. These products are in the milk in relatively small quantities, for example, lactoferrin. Removal of relatively small quantities does not substantially alter the characteristics of the milk being collected in the milk tank. In the case where caseins are removed the character of the milk would naturally be significantly different. If desired the filter medium in the cartridge may also be adapted to remove fats from the milk.

Another application of the invention is to remove some component which has deleterious side effects for human health. For example bovine milk has a different protein profile to that of human milk. It is particularly important in human infant formulations to have a milk more consistent with human milk. While in other situations some people (young and old) cannot tolerate lactose and need appropriately processed milk. The invention could be used to modify that milk in those cases by on-farm processing.

An advantage of the invention is to remove biochemical/biomedical species which are found in milk in quite small amounts. This would then allow the balance of milk to proceed through normal milk processing channels such as cheese making.

From time to time the cartridge(s) is/are removed and a new one substituted. The removable cartridge unit may be removed for direct treatment or stored in a refrigerated unit for subsequent collection and treatment to remove the required constituent.

Alternatively at the farm milk site the modular cartridge can be purged to remove the concentrated constituent(s) which is/are transported from further processing or use.

The cartridge unit can be sited at the milking claw or on the milk line pipes anywhere prior to the receiving tank. The manner in which it is fitted is such that it can be readily removed by hand to allow the milking system to be cleaned in the usual way.

Advantages of the invention are that if modular cartridges are sited at each milking claw, it is possible to selectively handle an animal or preferred animal production for example for testing. For example, a particular animal or animals could be treated, for example by injecting an antigen, to produce a particular antibody which is collected in the cartridge. This also provides for on-line testing or particular animals. This could be very important in assessing the milk output of an animal, or checking its health status.

Early removal of specific biomedical or biochemical products may minimise any loss of their activity. This loss of activity can occur during ordinary milk processing such as sterilisation and pasteurisation because of the processes through which the milk passes.

If the biochemical or biomedical constituent is removed prior to pasteurisation this would at least minimize or remove the possibility of any loss of (biological) activity. Protein denaturation is something which is important to avoid and one of the biggest factors in denaturation is heat. Most proteins start to denature when temperatures are raised above 40 degrees or so (the process is incremental with no absolute start point) clearly by avoiding the pasteurisation step the risk of denaturation can be avoided.

With protease removal earlier than they do with standard milk processing their action on other proteins, such as the caseins, would be eliminated. By quickly removing a peptide or protein or similar proteinaceous material, the deleterious effects of proteases (as could occur when milk, in toto, is left prior to processing) would be minimised. The cooling of milk which generally takes place in modern milking sheds does counteract the protease effect, since enzyme function is temperature dependent.

The invention can allow a farmer who is paid on the basis of milk proteins to monitor functions such as health of particular animals (by adapting the device to bind with certain key or single components. Also antibiotics in milk are unacceptable for milk processors and farms may be banned from passing on such milk. Those antibiotics would only be an issue if an animal had been ill and been subjected to a course of an antibiotic.

Another application, probably of limited use, would be for the processing of milk from transgenic animals where the animal is used as a "factory" to produce a specific protein. Attempts have been made to alter the genetic make-up of an animal so it produced an altered milk with some new or changed protein or other chemical composition. This has been a goal of other groups looking at transgenic cows for the production of lactoferrin.

Thus by this invention there is provided a process and apparatus by which specific milk constituents which may have biochemical or biomedical interest can be removed.

Where in the aforegoing description particular integers or process steps are referred to it is envisaged that their equivalents can be used as if they were set forth herein.

Particular examples of the invention have been described and it is envisaged that improvements and modifications can take place without departing from the scope thereof.

Enzyme source

Whole raw milk was collected by standard dairy farm procedures employing a Bou-Matic automatic milking system. Milk samples were provided by the University of Wisconsin-Madison Dairy Science Department, Dairy Cattle Center. Milk samples were processed immediately or stored (4°C) overnight prior to fractionation.

Peroxidase Assays

Lp activity wad determined by the 2,2'-azinobis(3-ethylbenzylthiazoline-6-sulfonic acid) ABTS) (Sigma Chemical Co., St. Louis, MO) method (1) using a final concentration of 5.0 mM ABBTS, 0.3 mM H_2O_2 in 10 mM sodium phosphate pH 6.7 buffer, measuring increased absorbance at 436nm ($\varepsilon^\circ = 2.93 \times 10^4$) (2).

Bio-Rex 70 Column chromatography

Milk was applied (35-37°C with stirring, 3 to 7 ml/min) to a 2.5 x 5.0 cm column (2.5 x 8.2 cm where indicated) packed with carboxymethyl (CM) Bio-Rex 70 (BR 70) 100-200 mesh particle size cation exchange matrix (product # 142-5842, Bio-Rad Laboratories, Inc., Hercules, CA) equilibrated with 10 mM sodium phosphate buffer pH 6.7 (buffer A). Unabsorbed proteins and fat were washed from the column with six column volumes buffer A + 01.M NaCl at 35°C. Adsorbed proteins were eluted either with a linear NaCl gradient in buffer A (0.1 to 1.0 M NaCl in 360 ml) or in two NaCl in buffer A steps: 1) 0.25 M NaCl, 100 ml; 2) 0.8 M NaCl. Elution was monitored at 280nm for protein and fractions were collected (6 to 14-ml as specified for each separation).

Lp fractions were pooled (Pool A) according to their peroxidase activity against ABTS; protein chromatogram; and SDS-PAGE determinations of purity against Lp standard (Sigma Chemical Co., St. Louis, MO). Lf fractions were pooled (Pool B) according to the protein chromatogram peak containing protein eluting at NaCl concentrations > 0.4 M NaCl and SDS-PAGE determinations of purity against Lf standard (Sigma Chemical Co., St. Louis, MO). Pools A and B were dialysed (4°C, overnight) separately into buffer A.

Bio-Rex 70, Maximum Dynamic Capacity for Lp and Lf

Milk (0.9 to 1.3 1) was applied (35-37°C with stirring, 3 to 4 ml/min, 7.0 ml fractions collected) to a 2.5 x 8.2 cm column packed with carboxymethyl (CM) Bio-Rex 70 100-200 mesh particle size cation exchange matrix and fractionated for Lp and Lf by NaCl gradient elution as described above.

Bio-Rex 70, Maximum Flow Rate (ml/min/cm²)

Milk (0.6 1) was applied (35-37°C with stirring, 3.5 to 7 ml/min, 13.5 ml fractions collected) to a 2.5 x 5.0 cm column packed with carboxymethyl (CM) Bio-Rex 70 100-200 mesh particle size cation exchange matrix. Fractionation of adsorbed Lp and Lf was achieved through step elutions with NaCl in buffer A as described above.

SP-5PW HPLC Purification of Lp and Lf

Dialysed pools A, Lp and B, Lf were fractionated independently by high pressure liquid chromatography (HPLC). The sample pools were centrifuged (15,000 x g, 30 min, 4°C), pellets were discarded, and the filtered (0.45 μm) supernatants, <50mg protein/load, were applied (8 ml/min, ambient temperature) to a 2.15 x 15 cm TSK SP-5PW column (Supelco Div. Rohm and Haas Co.). Unabsorbed proteins were washed from the column with buffer A until a stable baseline was observed. Adsorbed milk proteins were separated using a gradient HPLC system (Beckman Instruments Inc., Waldwick, NJ). Elution buffers were buffer A and buffer A + 1.0 M NaCl (buffer B). Chromatography was achieved with a continuous gradient of buffer A and B as follows: 100% A for 5 min, 100% A to 100% B in 25 min, 100% B for 5 min, 100% B to 100% A in 2 min, and 100% A for 15 min to equilibrate the column for the next sample. Elution was monitored at 280nm for protein and 412nm for heme-containing Lp. A flow rate of 8.0 ml/min was used and 8.0-ml fractions were collected. Fractions containing Lp or Lf were pooled as described above. Purified Lp and Lf were stored in buffer A at -70°C.

GibcoCel HB-2 and HG-2 Column Chromatography

Milk was applied (35-37°C with stirring, 3 to 4 ml/min, 7.0 ml fractions collected) to a 2.5 x 8.2 cm column packed with sulphopropyl (SP) GibcoCel HB-2 (beaded cellulose) or HG-2 (granular cellulose), 200 micro particle size cation exchange matrices products 30015-054 and 30017-054, respectively (Life Technologies, Inc., Gaithersburg, MD) equilibrated with buffer A. Fractionation of adsorbed Lp and Lf was achieved through gradient elution with NaCl in buffer A as described above for BR70.

3M Radial Pleated Filter Cartridge Loaded with Bio-Rex 70

Milk (4.0 l) was applied (35-37°C with stirring, ~3.0 litres/min) to a 7.0 x 25.0 cm radial pleated polypropylene filter (2.0 μm pore size) cartridge (3M Company, St. Paul, MN) loaded with 200 ml carboxymethyl (CM) Bio-Rex 70 100-200 mesh or 200-400 mesh particle size cation exchange matrix. The cartridge was housed in a model PSCL -S1 (Ametek, Sheboygan, WI) water filtration housing modified with a 1.0 x 25 cm dead end polypropylene dispersion manifold (20, 2 mm diameter perforations arranged linearly at 1.0 cm intervals) as the inlet line. The BR 70 Ion Exchange Filter (BRIEF) cartridge device was washed and equilibrated (2 x 3.0 l, 3.0 l/min, 5 min) with buffer A prior to loading with whole milk.

Fractionation of Lp and Lf was achieved by circulating the whole milk for 60 min. Followed by washing (3 x 2.0 l buffer A + 0.1 M NaCl) and NaCl step elutions (step 1,

2 x 2.0 l buffer A + 0.25 M NaCl; step 2, 2.0 l buffer A + 0.8 M NaCl) of adsorbed proteins from the BRIEF cartridge. Wash and elution circulation rates were approximately 3.0 l/min for 5.0 min, and solution temperatures were 35-37°C during washes and ambient during elutions.

Samples of the circulating milk were taken at several time points during loading and Lp activity measured by ABTS assay in order to monitor Lp removal by the BRIEF cartridge. Elution was monitored for Lp (ABTS assay) and both Lp and Lf by SDS-PAGE.

Electrophoresis

Protein purity and molecular weight were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Novex (San Diego, CA) pre-cast, 1.0 mm, 4-20% gradient, tris-glycine gels and/or a Pharmacia Phast System (Piscataway, NJ) using 10% to 15% gradient polyacrylamide gels and a Sigma SDS-6H standard (Sigma Chemical Co., St. Louis, MO) containing six proteins in the 29,000 to 205,000 mol wt range, straining with Coomassie Brilliant Blue G250.

Protein

Protein concentrations were determined by the bicinchoninic acid (BCA) method (3) with bovine serum albumin, Cohn fraction V (Sigma Chemical Co., St. Louis, MO) as standard. Additionally, crude samples were analysed at the University of Wisconsin-Madison Center for Dairy Research for total nitrogen by the Kjeldahl method (4), and true protein by trichloroacetic acid (TCA) precipitation of total protein (5) and BCA assays of the precipitated protein.

Fat

Fat concentration was determined at the University of Wisconsin-Madison Center for Dairy Research using the Mojonnier Method (6).

RESULTS

A. Capture and Separation of Lp and Lf from Whole Milk Using BR 70 Cation Exchanger

1. Results of BR 70 runs #10 and #12, fractionate 100ml whole milk, 3ml/min, 2.5 x 8.2 cm 40 ml column.

Lp and Lf were successfully captured and separated from whole milk employing

BR 70 (Fig. 2). In these experiments, BR 70 was used to capture Lp and Lf from whole milk by column chromatography. The adsorbed Lp and Lf were separated by NaCl gradient elution from 0 to 1.0 M concentration.

SDS-PAGE analysis of samples taken at several stages of milk fractionation runs 10 and 12 are shown in Figs. 3 and 4, respectively. Careful comparison of lane 1, milk feed stream prior exposure to the BR 70; versus lane 2, milk feed stream after exposure to BR 70, reveals Lp and Lf have been completely captured and removed by the fractionation procedures. These target proteins were then selectively eluted by exposure of BR 70 to a NaCl gradient. The chromatographic fractionation resulted in separation and enrichment of Lp (lanes 3-5, Fig. 2; lanes 3 and 4 Fig 4) and Lf (lanes 6 and 7, Fig. 3; lanes 5 and 6, Fig. 4) as compared to the crude whole milk feed stream (lane 1, Figs. 3 and 4).

Recovery of Lp as measured by ABTS assay was > 80% for run #12 with 435 international units (IU) applied to the BR 70 and 356 IU recovered in fractions 26-31 (Fig. 2).

Critical elements of the fractionation identified by these experiments were:

a. Whole milk can be fractionated by packed bed column chromatography employing acrylic based supports such as BR 70 (Fig. 2).

b. Whole milk feed stream temperatures of 35-37°C prevent fouling of the support by insoluble fat due to fat melting and decreased viscosity at the elevated temperature.

c. Selective removal of target proteins Lp and Lf was possible with minimal decreases in total milk proteins (Figs. 3 and 4).

d. High recoveries of target proteins were demonstrated with measured Lp yields of >80%.

2. Results of BR 70 runs #13 and #14, dynamic capacity experiments.

Maximum capacity of BR 70 for Lp and Lf was determined in fractionation runs 13 and 14 Figs. 5 and 7, respectively. Run 13 Fig. 5 chromatogram shows the results of a 930 ml whole milk fractionation by BR 70. This experiment was used to determine maximum resin loading capacity for whole milk while maintaining optimum separation and high yields of Lp and Lf. Final HPLC purification of these proteins is discussed below (section III, B). Resolution of Lp and Lf was maintained as shown in Fig. 5 with Lp eluting at approximately 0.25 M NaCl concentration and Lf eluting at approximately 0.5 M NaCl concentration.

Run 14 Fig. 7 chromatogram shows the results of a 1,280 ml whole milk fractionation by BR 70. This experiment was used to determine maximum resin loading capacity for whole milk without concern for high yield or optimum separation. Whole milk was loaded onto the BR 70 column until Lp activity was measured in the flow through fractions. Unabsorbed proteins and fat were washed from the column and adsorbed proteins were eluted as described above. Comparison of chromatograms for run 13 (Fig. 5) and run 14 (Fig. 7) revealed a notable difference in the ratio of Lf to Lp based on protein absorbance at 280nm. Peak area for Lf was 1.3 times greater than peak area for Lp in run 13 but in run 14, Lf peak area was 1.9 times that of Lp. One possible explanation for the different ratios of Lf to Lp is, in run 14 the load of Lf was great enough to displace Lp from the BR 70. Lf has a greater affinity and adsorbs more strongly to BR 70 due to its higher pI. Lf's higher Br 70 affinity is demonstrated by its elution at 0.5 M NaCl versus the 0.25 M NaCl concentration necessary to elute Lp.

SDS-PAGE analysis of samples taken at several stages of milk fractionation run 13 are shown in Fig. 6. Careful comparison of lane 1, milk feed stream prior exposure to the BR 70; versus lane 2, milk feed stream after exposure to BR 70, reveals Lp and Lf have been completely captured and removed by the BR 70 fractionation procedures. These target proteins were then selectively eluted by exposure of BR 70 to a NaCl gradient. The chromatographic fractionation resulted in separation and enrichment of Lp (lane 3, Fig. 6) and Lf (lanes 4, Fig. 6) as compared to the crude whole milk feed stream (lane 1, Fig. 6).

Recovery of Lp as measured by ABTS assay was ~85% for run #13 with 2,967 IU applied to the Br 70 and 2,509 IU recovered in fractions 6-13 (Fig =. 5). Recovery of Lp was ~62% for run #14 with 4,234 IU applied to the BR 70 and 2,639 IU recovered in fractions 8-17 (Fig. 7).

These examples establish:

a. Calculated capacity of BR 70 for Lp and Lf from a whole milk feed stream is approximately 43 litres whole milk per kilogram BR 70 or 4.3 g Lf and 1.3 g Lp. Calculation based on Lp and Lf concentrations from literature.

b. BR 70 is not fouled by excessive whole milk loads up to its maximum capacity for Lp and Lf.

c. High fat concentrations present in whole milk do not interfere with adsorption and chromatographic fractionation of Lp and Lf by BR 70.

d. Good separation of Lp and Lf as well as yields of 60-85% for Lp can be achieved at or near BR 70's maximum loading capacity.

e. Under conditions of excessive whole milk loading, Lf displaces Lp from BR 70 due to its higher affinity for the chromatographic support (Lp > Lf >> adsorption to BR 70).

3. Results of Bio-Rex 70 runs #15 and 16, flow rate experiments.

Maximum flow rate for BR 70 was determined in fractionation runs 15 and 16 Figs. 8 and 10, respectively. Run 15 (Fig. 8) chromatogram shows the results of a 600 ml whole milk fractionation by BR 70 at a flow rate of 3.5 ml/min (0.71 ml/min/cm^2), and the corresponding SDS-PAGE analysis (Fig. 9) of samples taken at several stages of the fractionation. Run 16 (Fig. 10) chromatogram shows the results of a 600 ml whole milk fractionation by BR 70 at a flow rate of 7.0 ml/min (1.43 ml/min/cm^2), and the corresponding SDS-PAGE analysis (Fig. 11) of samples taken at several stages of the fractionation. This experiment was used to determine maximum flow rate for whole milk fractionation by BR 70 while maintaining resolution and high yields of Lp and Lf.

Comparisons of chromatograms (Figs. 8 and 10) and gels (Figs. 9 and 11) demonstrated that reasonably high flow rates are not deleterious to resolution or quality of the Lp and Lf. Additionally, step elution was used instead of a NaCl gradient. Lp was eluted by 0.25 M NaCl and Lf by 0.8M NaCl resulting in a more concentrated product than with a gradient elution method as the target proteins can be eluted quickly in a smaller volume and are not diluted by the gradient. Finally, flow rates . 1.43 ml/min/cm^2 are not recommended when fractionating whole milk by column chromatography with BR 70 in a packed bed format. Higher flow rates resulted in resin bed fractures, channelling of the feed stream, inferior resolution and decreased yields.

SDS-PAGE analysis of samples taken at several stages of milk fractionation runs 15 and 16 are shown in Figs. 9 and 11, respectively. Careful comparison of lane 1, milk feed stream prior exposure to the BR 70; versus lane 2, milk feed stream after exposure to BR 70, reveals Lp and Lf have been completely captured and removed by the BR 70 fractionation procedures. These target proteins were then step eluted by exposure of BR 70 to NaCl steps of 0.25 M and 0.8 M. The chromatographic fractionation resulted in separation and enrichment of Lp eluted by the 0.25 M salt concentration (lane 3, Figs. 9 and 11) and Lf eluted by the 0.8 M salt concentration (lanes 4, Figs. 9 and 11) as compared to the crude whole milk feed stream (lane 1, Fig. 9 and 11).

Analysis of whole milk for total protein and fat gave results of 3.144% protein and 3.984% fat before exposure to BR 70 fractionation procedures and 3.115% protein and 3.907% fat after exposure to the BR 70 fractionation. Therefore, very minimal change in the overall protein and fat content resulted from the fractionation and removal of Lp

and Lf by BR 70. Protein was reduced only .029 % and fat by .077% after exposure to the BR 70.

Recovery of Lp as measured by ABTS assay was ~95% for run #16 with 1,221 IU applied to the BR 70 and 1,159 IU recovered in fractions 2 and 3 (Fig. 10).

Critical elements of the fractionation identified by these experiments were:

a. Maximum flow rate for BR 70 in a packed bed column format for fractionating Lp and Lf from whole milk was 1.43 ml/min/cm^2.

b. Lp and Lf can be eluted by 0.25 M and 0.8 M NaCl steps, respectively.

c. Protein and fat content were decreased <0.1% through BR 70 fractionation procedures.

B. Final Purification by SP-5PW HPLC

The results of a typical purification of Lp from whole milk are shown in Table 1.

TABLE 1 - Purification table for Lp from Bovine Milk

Purification Step	Volume ml	Protein Conc. (Mg/ml)	Units (IU/ml)	Total Units	Specific Acitivity	Yield (%)	Fold Purification
Whole Milk	930	22.60	3.2	2,976	0.14	100	1.0
BioRex 70	57	0.99	35.2	2,006	35.60	67	254
Dialysis	52	0.65	28.8	1,498	44.30	50	316
SP 5PW HPLC	16	0.95	90.0	1,440	94.70	48	676

The majority of the purification was achieved through the two cation exchange chromatography steps. The fractionation by BR 70 (Fig. 5) gave a 254 fold purification and the Lp was purified an additional 422 fold by dialysis and SP 5PW HPLC (Fig. 12). Overall the Lp was purified 676 fold by these procedures and 48% of the initial activity measured in the whole milk was recovered as pure protein.

Quantitative recovery and purity data for Lf was not determined, because a functional assay was not available. However, Lf was purified according to its BR 70 (Fig. 5) and SP 5PW (Fig. 13) elution times and SDS-PAGE analysis as compared to pure Lf standard (Sigma Chemical Co.).

SDS-PAGE analyses of samples taken at several stages of the fractionation protocol are shown in Figs. 14-16. At least nine other major milk proteins as well as the faint Lp and Lf bands can be identified in the whole milk starting material (Fig. 14, lane

1). BR 70 cation exchange effectively removed the majority of the unwanted milk proteins from the Lp (Fig. 14, lane 3) and Lf (Fig. 14, lane 4) fractions. The SP 5PW HPLC cation exchange procedure yielded highly purified preparations of Lp (Fig. 13, lane 5; and Fig. 15, lanes 3 and 4) and Lf (Fig. 14, lane 8: and Fig. 16, lanes 4-6) seen as a single protein bands by SDS-PAGE.

These examples establish:

a. Lp and Lf can be selectively removed from whole milk by BR 70 in a packed bed column format and a 254 fold enrichment of Lp was achieved in a single chromatographic step.

b. Semi-pure fractions of Lp and Lf eluted from the BR 70 column could be easily processed to the highly purified proteins by SP 5PW HPLC.

Test of a modular BR 70 ion exchange filter cartridge device for milk fractionation.

Lp and Lf were successfully captured and separated from whole milk employing a cartridge (Figs. 17 and 18) (a 3M proprietary cartridge - BRIEF™ cartridge). In these experiments, BR 70 was used in a filter cartridge device to capture Lp and Lf from 4.0 l of the whole milk at a high flow rate (3.0 l/min). In contrast to previously described BR 70 fractionations where small volumes (<1.0l), low flow rates (≤7.0 ml/min), and a packed bed column format were used, the BRIEF cartridge device demonstrates the successful application of another format for the fractionation of larger volumes of whole milk at higher flow rates. However, the BR 70 packed bed column format could certainly be scaled up to accommodate higher flow rates and larger volumes.

Circulation of the whole milk feed stream was necessary in order to capture the target proteins (Figs. 17 and 18). After only 2 min, 55% of the Lp was captured from the circulating milk, and 89% was removed in 10 min. Although Lf depletion during the milk circulation through the BRIEF cartridge was not monitored, capture of Lf was accomplished as seen in the SDS-PAGE analysis described below.

SDS-PAGE analysis of samples taken at several stages of BRIEF cartridge milk fractionation is shown in Fig. 18. Careful comparison of lane 1, milk feed stream prior exposure to the BRIEF cartridge; versus lane 2, milk feed stream after exposure, reveals Lp and Lf have been completely captured and removed by the fractionation procedures. These target proteins were then step eluted by exposure of the cartridge to NaCl steps of 0.25 M and 0.8 M. The fractionation resulted in separation and enrichment of Lp eluted by ~~the 0.25 M salt concentration (lane 3, Fig. 18) and Lf eluted by the 0.8 M salt~~

by the 0.25 M salt concentration (lane 3, Fig. 18) and Lf eluted by the 0.8 M salt concentration (lane 4, Fig. 18) as compared to the crude whole milk feed stream (lane 1, Fig. 18).

Recovery of Lp as measured by ABTS assay was 70% with 10,448 international units (IU) applied to the BRIEF cartridge and 7,283 IU recovered in the 0.25 M NaCl elutions.

These examples establish:

a. Whole milk can be fractionated employing the BRIEF cartridge (Fig. 17).

b. Fractionation can be achieved at high flow rates allowing large volumes to be processed quickly.

c. In addition to fractionation by ion exchange, the BRIEF cartridge incorporates a feed stream filtration (2.0 μm) due to the radial pleated filter employed as a support matrix for the BR 70.

d. Although recirculation of the feed stream was necessary for maximum Lp and Lf capture in these experiments, several cartridges could be arranged in series or a resin with higher affinity for the target protein(s) could be loaded into the filter cartridge allowing efficient capture in a single pass.

e. If complete capture of Lp and Lf is not critical, one third of the Lp can be captured in a single cycle and over half of the Lp is captured after two cycles.

Whilst preferred forms of the present invention have been described in relation to the endogenous proteins lactoperoxidase (Lp) and lactoferrin (Lf) as previously set forth the terms "biomedical or biochemical constituents of milk" or "endogenous protein(s) of milk" includes any one or more of the following

- beta-lactoglobulin
- alpha-lactalbumin
- serum albumin
- immunoglobulins
- lactoferrin
- lactoperoxidase
- gamma casein
- proteose peptone
- glycoproteins
- beta-casein
- alpha-casein (and the two sub-classes of alpha-S1-casein and alpha-S2-casein)
- kappa-casein

- whey acidic protein
- beta-macroglobulin
- trypsin inhibitor protein
- protease
- growth hormone

Obviously if not restricted to bovine animals other substituents may be more prevalent. Similarly, if a "transgenic" animal is involved the term may also extend to

- clotting factor IX
- alpha-1 antitrypsin
- tissue plasminogen activator
- follicle simulating hormone
- growth hormone
- interleukin-2

It is to be noted however that the proteins of choice set out above are ones expected to be present in bovine milk although the levels of each species can vary according to the physiological condition of the cow. For example, at the onset of lactation, the colostrum (immature milk) would be expected to contain higher levels of antibodies and the trypsin inhibitor protein. Those species may disappear at latter stages of the cows lactation sequence.

The human milk is devoid of beta-lactoglobulin, which is one reason why there could be commercial interest in removing beta-lactoglobulin from bovine milk (to avoid possible immunological responses in children using bovine-based milk supplements).

The removal of growth hormone (commonly abbreviated as either BST or BGH) from cows milk can be carried out using the present invention. This may have appeal since there is considerable consumer opposition to the use of milk from animals which have been treated with either BST or BGH. Thus removal of both naturally occurring BGH and any enhanced level of BGH from cows milk may render marketable milk products that might otherwise have had a stigma attached to them owing to the milk yield having been enhanced by the use of the protein.

As far as transgenic animals are concerned human growth hormone as well as many other proteins of interest can be produced using the animal as a factory. Such technology is well known.

Whilst the preferred forms of the present invention in relation to lactoperoxidase and lactoferrin have been described primarily with a collection means reliant upon ion exchange technology other technologies as previously indicated are possible. It is to be appreciated that many different chromatographic supports are available and at least one

matrix is available to match each of the proteins mentioned above.

It should be aware however that whilst the procedure has been shown to be viable with an ion exchange resin, the molecular filtering device may employ ultrafiltration, a chromatographic resin, an ion exchange resin, a molecular sieve resin, an antibody-linked resin, a metal affinity resin or a chromatographic or other impregnated paper or other filter medium.

Whilst a number of different means or matrices have been discussed it is appropriate also to mention some of the different options available.

The resin used in the lactoferrin work (Bio-Rex 70) is classified as a 'weak cationic' resin. The material is an acrylic-based resin on which the functional groups are carboxylic moieties eg; $R\text{-}COO^-$.

(Note: The Bio-Rex resin is actually a slightly refined fraction of resin obtained in bulk from Rohm & Haas -- those latter Rohn & Haas resins have the trade name of "Amberlites". The Bio-Rex 70 material is at least similar to Rohm & Haas IRC-50. "Strong cationic" resins are another class of resins in which the functional group is often a sulphonic acid moiety. Other larger producers of these types of resins are the Dow Chemical Company and the Mitsubishi Chemical Company -- they in turn have their own trade names, like Dowex and Duolite).

Since both lactoferrin and lactoperoxidase proteins have a nett positive charge they can bind to a weak cationic ion exchange resin. We actually tested a number of weak cationic resin (including one based on cellulose, rather than acrylic) -- which is manufactured in Nelson, New Zealand, its trade name is "Indion" and found the Bio-Rex 70 to be the best candidate.

For many of the other proteins naturally found in milk, there net charge is negative and so likely candidates for ion exchange chromatography (at least) -- if that was the desired option for their removal -- would be anionic resins. These may be either "weak" of "strong" anionic materials such as the Amberlite range (IRA's) where the backbone would be a polystyrene and the functional groups either quaternary ammonium or polyamine.

(Note: The nett charge of a protein will alter according to the pH of the surrounding environment. This phenomenon is exploited in protein purification research by examining, or determining, the pH at which the protein has a nett neutral charge -- its pI value. Once that is known one can more easily select anionic versus cationic resins for the purification protocol. In all cases the nett charge of a protein to which we are referring to, is that found at the normal physiological pH of milk.)

It would be more likely in fact that for small (commercially valuable) milk proteins

they may be better removed by affinity systems which have an antibody (to the target protein) covalently bound to some chromatographic matrix. The beauty of the ion exchange system, we used for lactoferrin and lactoperoxidase, is that IRC-50 can be obtained in bulk quantities and so at a very low cost. For on-farm processing (as we had always envisaged for this technology) this low cost approach has obvious commercial, and practical, appeal.

WHAT WE CLAIM IS:

COGNATE Nos 31-xx

1. A process whereby specific targeted biomedical or biochemical constituents of milk are removed during farm milk handling, the process including:

siting at a farm milking plant means for removing a targeted milk constituent or constituents;

passing milk past or through the means; and

collecting the targeted milk constituent(s) for analysis or further processing.

2. Milking apparatus for milking animals and which includes provision for removing targeted milk constituent(s) from the milk stream, said apparatus including

means to pass milk freshly milked from an animal or a group of animals through a matrix capable of collecting the targeted milk constituent(s), and

means to allow subsequent collection of the constituent(s) containing matrix or the subsequent elusion harvesting of the constituent(s) from the matrix.

3. Apparatus of claim 1 wherein said matrix is contained within a removable cartridge.

4. Apparatus of claim 2 or 3 wherein said matrix removes the targeted milk constituent(s) by filtering or affinity.

5. Apparatus as claimed in claim 4 wherein said matrix collects targeted milk constituent(s) by using any one or more of ultra filtration, a chromatographic resin, an ion exchange resin, a molecular sieve resin, an antibody linked resin, a metal affinity resin, a chromatographic or other impregnated paper or other filter medium.

6. A process of claim 1 wherein the targeted milk constituent(s) is at least an endogenous milk protein, said process including a method of isolating one or both of the endogenous milk proteins lactoperoxidase (Lp) and lactoferrin (Lf), said method comprising

presenting the whole milk of one or several animals on or adjacent the milking site of such a mammal or mammals to a matrix with an affinity for the desired endogenous protein to allow the attachment thereof to such matrix, and

thereafter eluting or otherwise removing the desired endogenous protein or proteins from such matrix.

7. A process of claim 1 wherein the targeted milk constituent(s) is at least an endogenous milk protein, said process including a method of isolating an endogenous milk protein from a milk source which comprises

(i) substantially at the body temperature of the mammal from which the milk is being sourced presenting the whole milk to a matrix with an affinity for the desired endogenous milk protein, and

COGNATE filed 31 JUL 06

(ii) thereafter eluting or otherwise removing the desired endogenous protein from the matrix.

8. A process of claim 6 or 7 wherein the presenting is at a temperature at which the milk fat remains liquid.

9. A process of claim 6 wherein said isolation is of both endogenous proteins and a selective eluting is used to isolate each protein from the other.

10. A process of any one of claims 6 to 9 wherein the matrix is a packed bed column and the matrix is an appropriate matrix for packed bed column chromatography.

11. A process of claim 10 wherein said packed bed column employs an acrylic based support.

12. A process of claim 11 wherein said acrylic based support is BR70 as hereinbefore defined.

13. A process of any one of claims 6 to 12 wherein the whole milk is presented to the matrix at a temperature within the range of 10°C of the mammals blood temperature and most preferably from 35 to 37°C.

14. A process of any one of claims 6 to 13 wherein said eluting is carried out at a temperature within the range of from 35 to 37°C.

15. A process of any one of claims 6 to 14 wherein each individual milk feed from a single mammal is presented to a dedicated matrix dedicated to either that mammal or a series of mammals being milked at that station.

16. A process of any one of claims 6 to 15 wherein eluting is with a sodium chloride solution.

17. A process of claim 16 wherein Lp is eluted with about 0.25M sodium chloride solution.

18. A process of claim 16 or 17 wherein Lf is eluted with about a 0.8M NaCl solution.

19. A process of any one of claims 1, and 6 to 18 when performed substantially as hereinbefore defined with or without reference to any one or more of the accompanying drawings.

20. A protein isolated by a process of any one of the preceding claims.

DATED THIS 28th DAY OF July 1998
A.J. PARK & SON
PER
AGENTS FOR THE APPLICANT

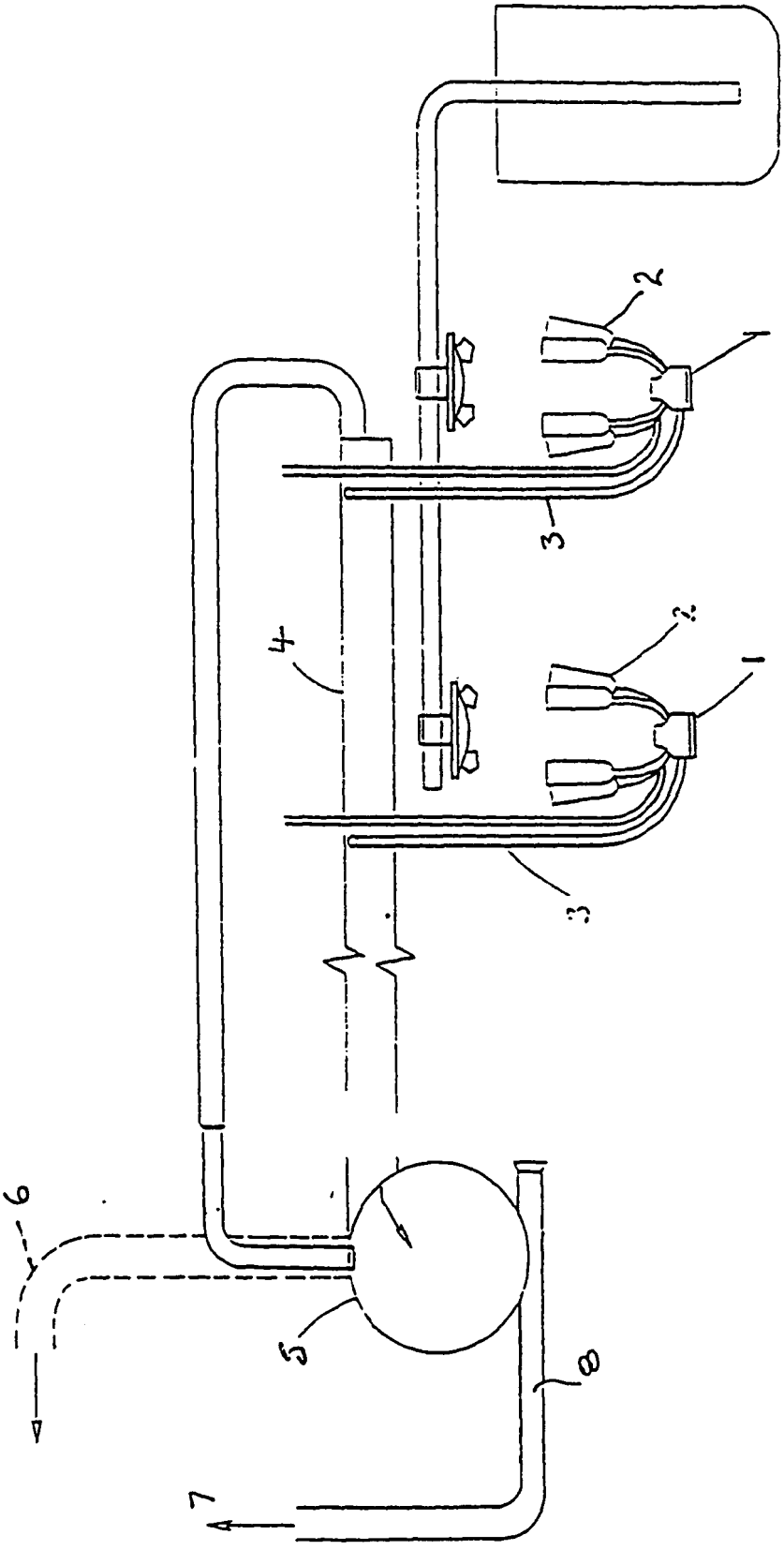
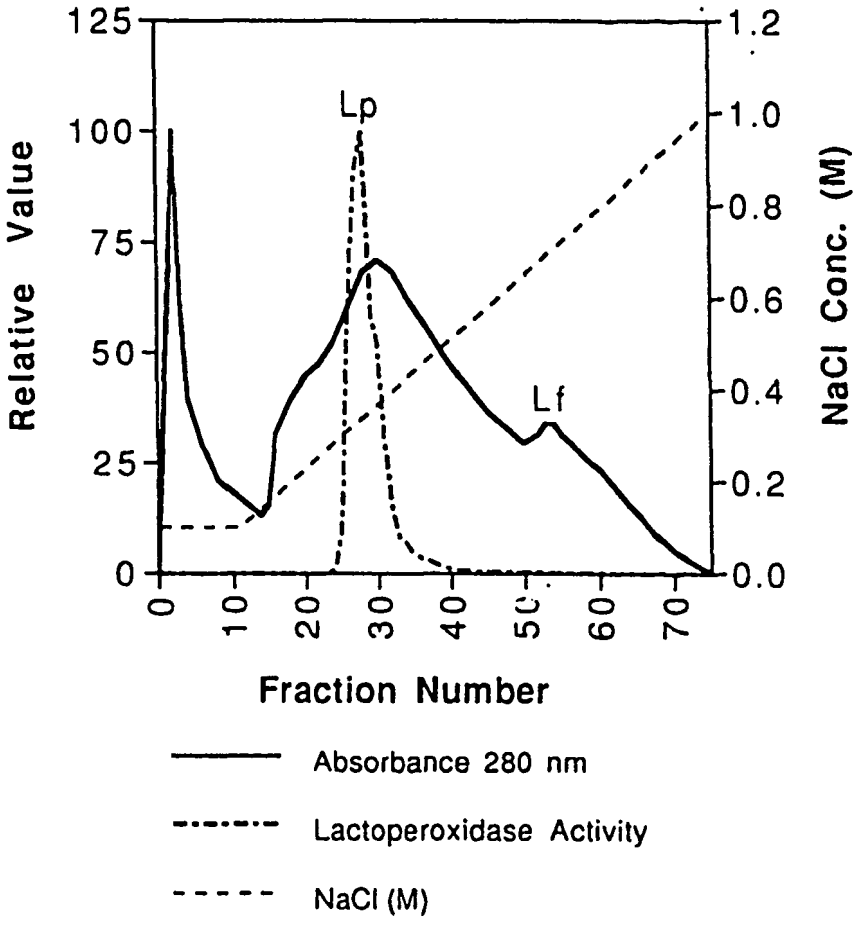


Figure 1.

Figure 2

Biorex 70 Chromatography of Whole Milk
Run #12



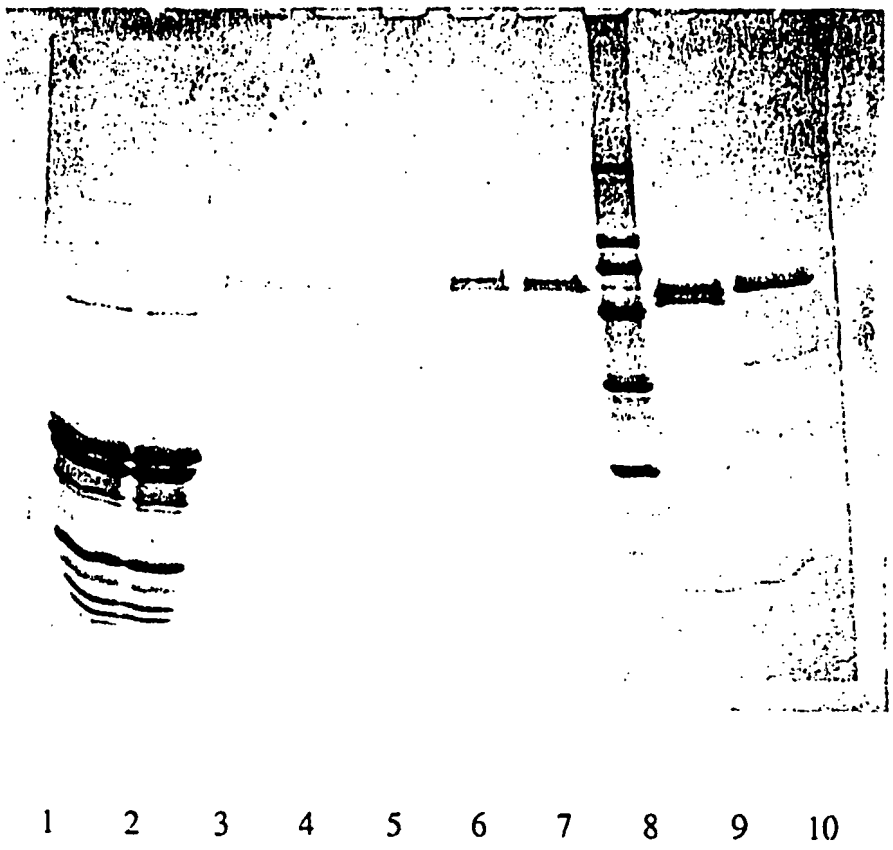


Figure 3 . Fractions at several stages of the purification: lanes 1, whole milk = BR 70 load; 2, whole milk after BR 70 exposure; 3-5, Lp peak fractions 17, 18, and 22, respectively, from BR 70 column run 10 (chromatogram not shown); 6 and 7, Lf peak fraction 43 and 47, respectively, from BR 70 column run 10; 8, SDS 6H molecular weight markers top to bottom: rabbit muscle myosin 205 kDa, E. coli β-galactosidase 116 kDa, rabbit muscle phosphorylase b 97.4 kDa, bovine albumin 66 kDa, egg albumin 45 kDa, and bovine carbonic anhydrase 29 kDa; 9, Bovine Lp standard protein (Sigma Chemical Co.) 77.5 kDa; 10, Bovine Lf standard protein (Sigma Chemical Co.) 83.1 kDa.

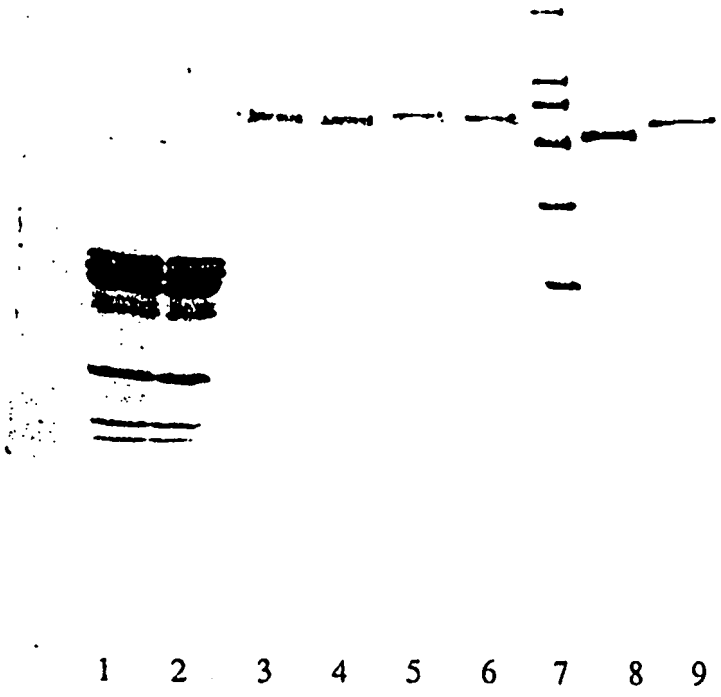
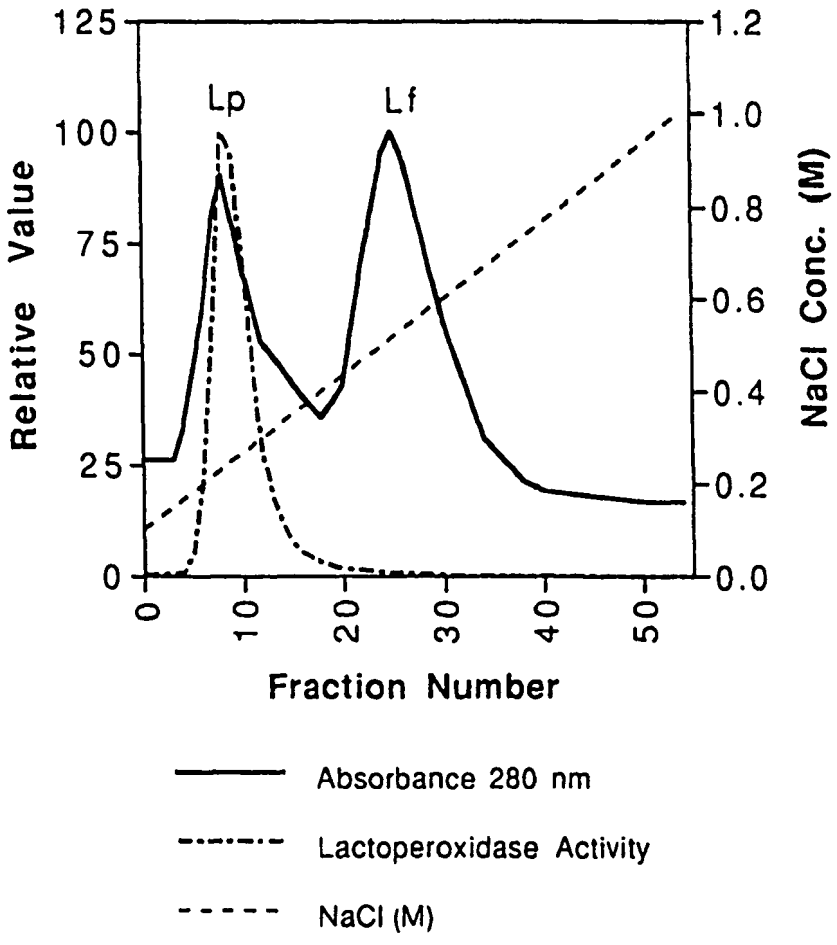


Figure 4 Fractions at several stages of the purification: lanes 1, whole milk = BR 70 load; 2, whole milk after BR 70 exposure; 3 and 4, Lp peak fractions 27 and 28, respectively, from BR 70 column run 12 (Fig. 1); 5 and 6, Lf peak fraction 54 and 55, respectively, from BR 70 column run 12; 7, SDS 6H molecular weight markers top to bottom: rabbit muscle myosin 205 kDa, E. coli β-galactosidase 116 kDa, rabbit muscle phosphorylase b 97.4 kDa, bovine albumin 66 kDa, egg albumin 45 kDa, and bovine carbonic anhydrase 29 kDa; 8, Bovine Lp standard protein (Sigma Chemical Co.) 77.5 kDa; 9, Bovine Lf standard protein (Sigma Chemical Co.) 83.1 kDa.

Figure 5

Biorex 70 Chromatography of Whole Milk
Run #13



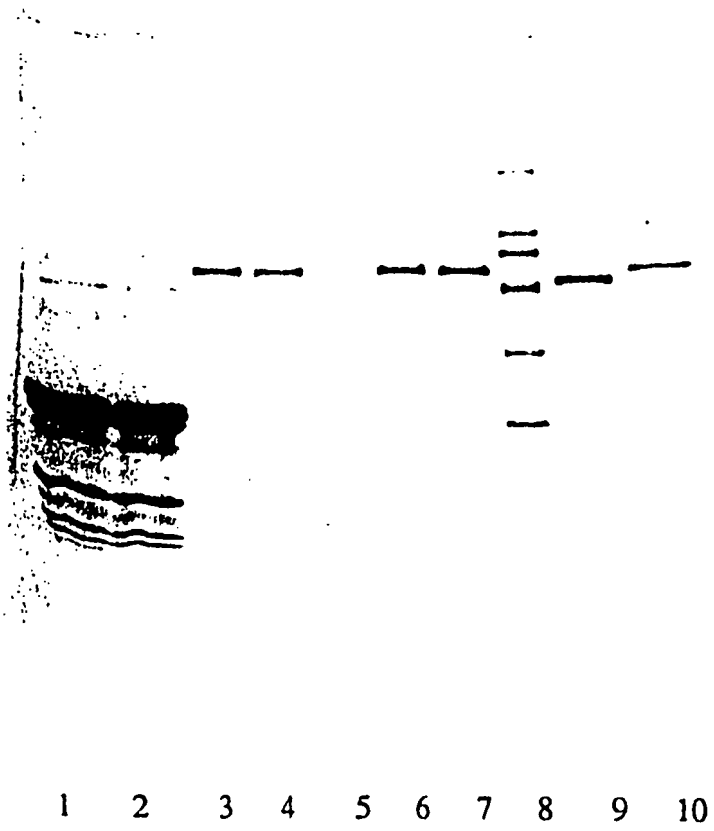


Figure 6 Fractions at several stages of the purification: lanes 1, whole milk = BR 70 load; 2, whole milk after BR 70 exposure; 3 and 4, Lp peak fractions 8 and 9, respectively, from BR 70 column run 13 (Fig. 4); 5, fraction 16 from BR 70 run 13; 6 and 7, Lf peak fraction 25 and 26, respectively, from BR 70 column run 13; 8, SDS 6H molecular weight markers top to bottom: rabbit muscle myosin 205 kDa, E. coli β-galactosidase 116 kDa, rabbit muscle phosphorylase b 97.4 kDa, bovine albumin 66 kDa, egg albumin 45 kDa, and bovine carbonic anhydrase 29 kDa; 9, Bovine Lp standard protein (Sigma Chemical Co.) 77.5 kDa; 10, Bovine Lf standard protein (Sigma Chemical Co.) 83.1 kDa.

Figure 7

Biorex 70 Chromatography of Whole Milk
Run #14

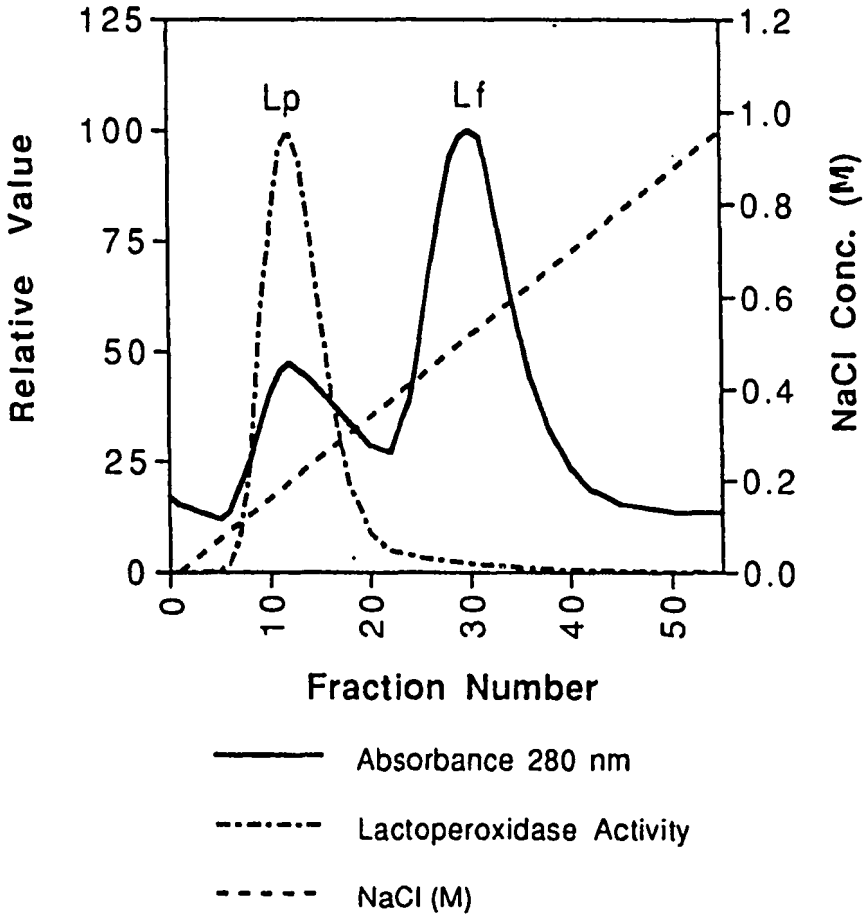
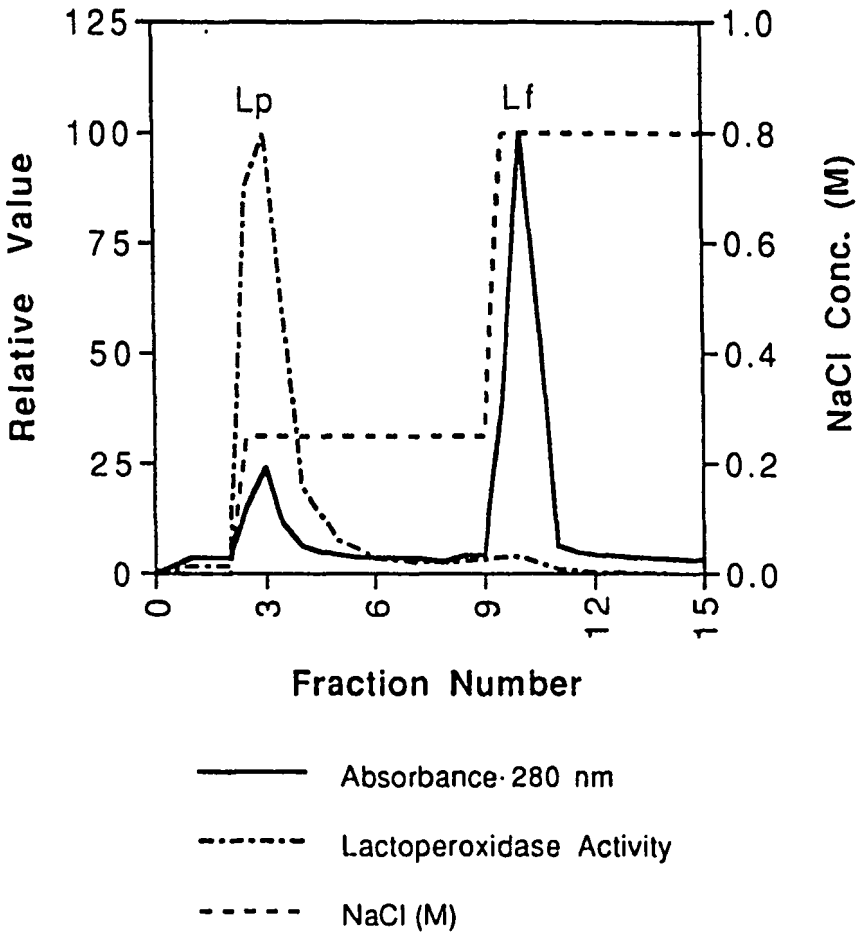
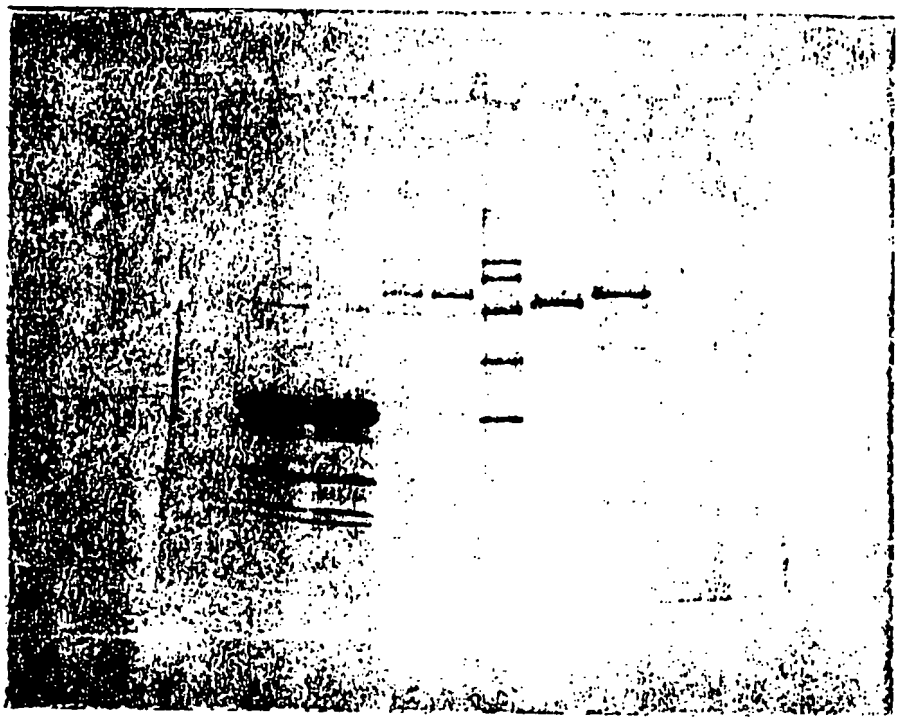


Figure 8

Biorex 70 Chromatography of Whole Milk
Run #15



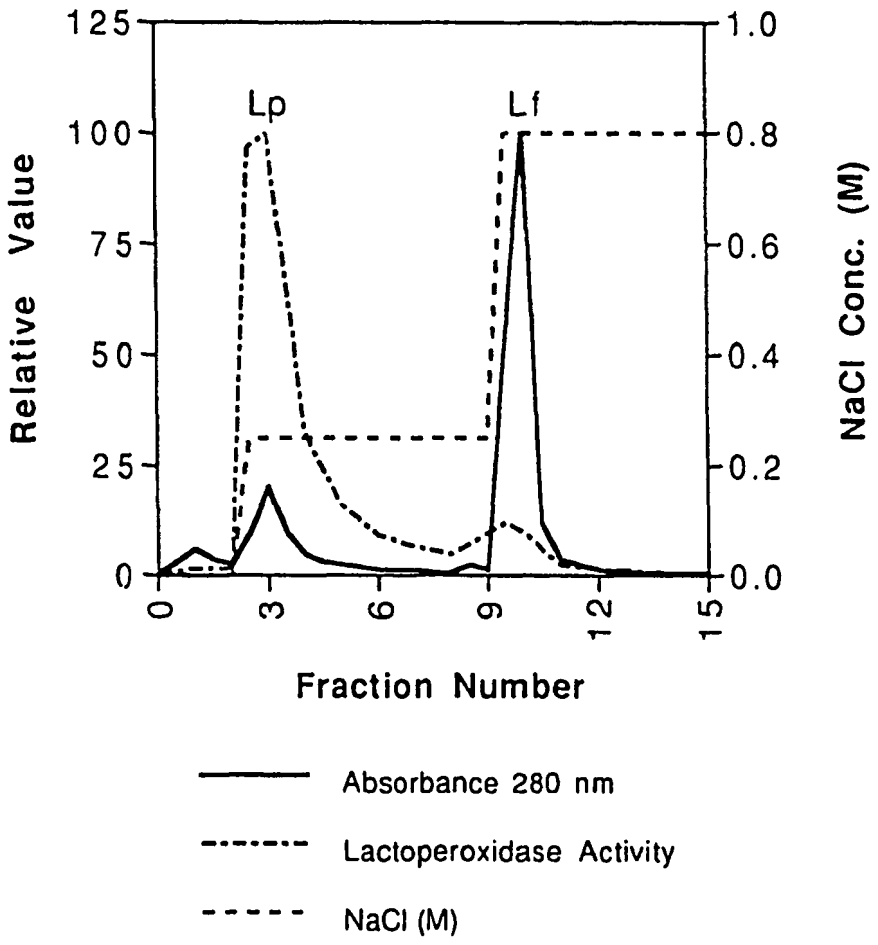


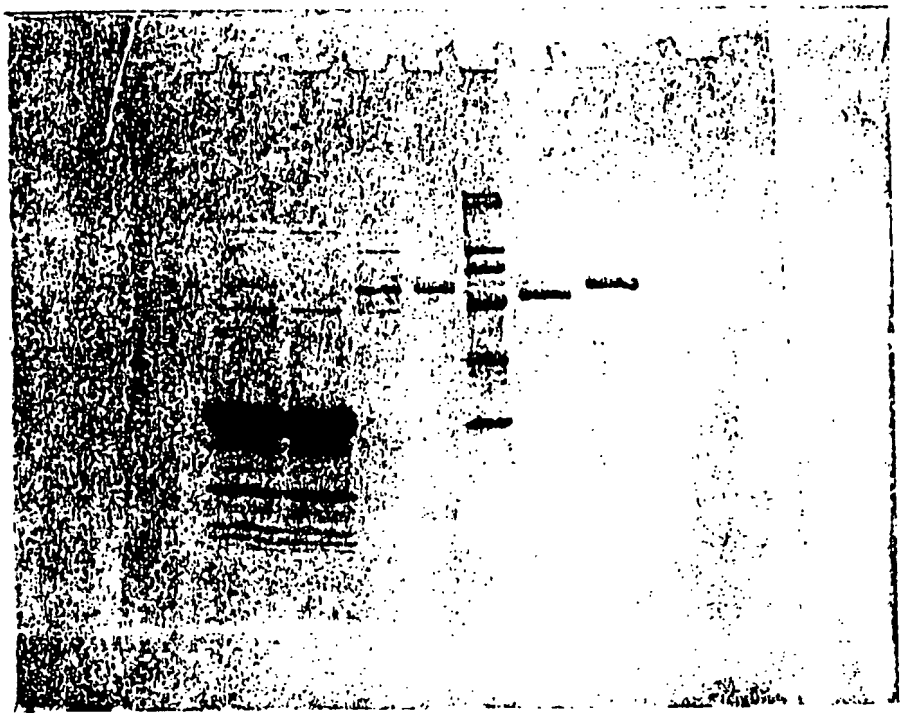
1 2 3 4 5 6 7

Figure 9 Fractions at several stages of the purification: lanes 1, whole milk = BR 70 load; 2, whole milk after BR 70 exposure; 3, Lp peak fractions pooled 2 and 3 from BR 70 column run 15 (Fig. 7); 4, Lf peak fractions pooled 9 and 10, from BR 70 column run 15; 5, SDS 6H molecular weight markers top to bottom: rabbit muscle myosin 205 kDa, E. coli β-galactosidase 116 kDa, rabbit muscle phosphorylase b 97.4 kDa, bovine albumin 66 kDa, egg albumin 45 kDa, and bovine carbonic anhydrase 29 kDa; 6, Bovine Lp standard protein (Sigma Chemical Co.) 77.5 kDa; 7, Bovine Lf standard protein (Sigma Chemical Co.) 83.1 kDa.

Figure 10

Biorex 70 Chromatography of Whole Milk
Run #16





1 2 3 4 5 6 7

Figure 11 Fractions at several stages of the purification: lanes 1, whole milk = BR 70 load; 2, whole milk after BR 70 exposure; 3, Lp peak fractions pooled 2 and 3 from BR 70 column run 16 (Fig. 9); 4, Lf peak fractions pooled 9 and 10, from BR 70 column run 16; 5, SDS 6H molecular weight markers top to bottom: rabbit muscle myosin 205 kDa, E. coli β-galactosidase 116 kDa, rabbit muscle phosphorylase b 97.4 kDa, bovine albumin 66 kDa, egg albumin 45 kDa, and bovine carbonic anhydrase 29 kDa; 6, Bovine Lp standard protein (Sigma Chemical Co.) 77.5 kDa; 7, Bovine Lf standard protein (Sigma Chemical Co.) 83.1 kDa.

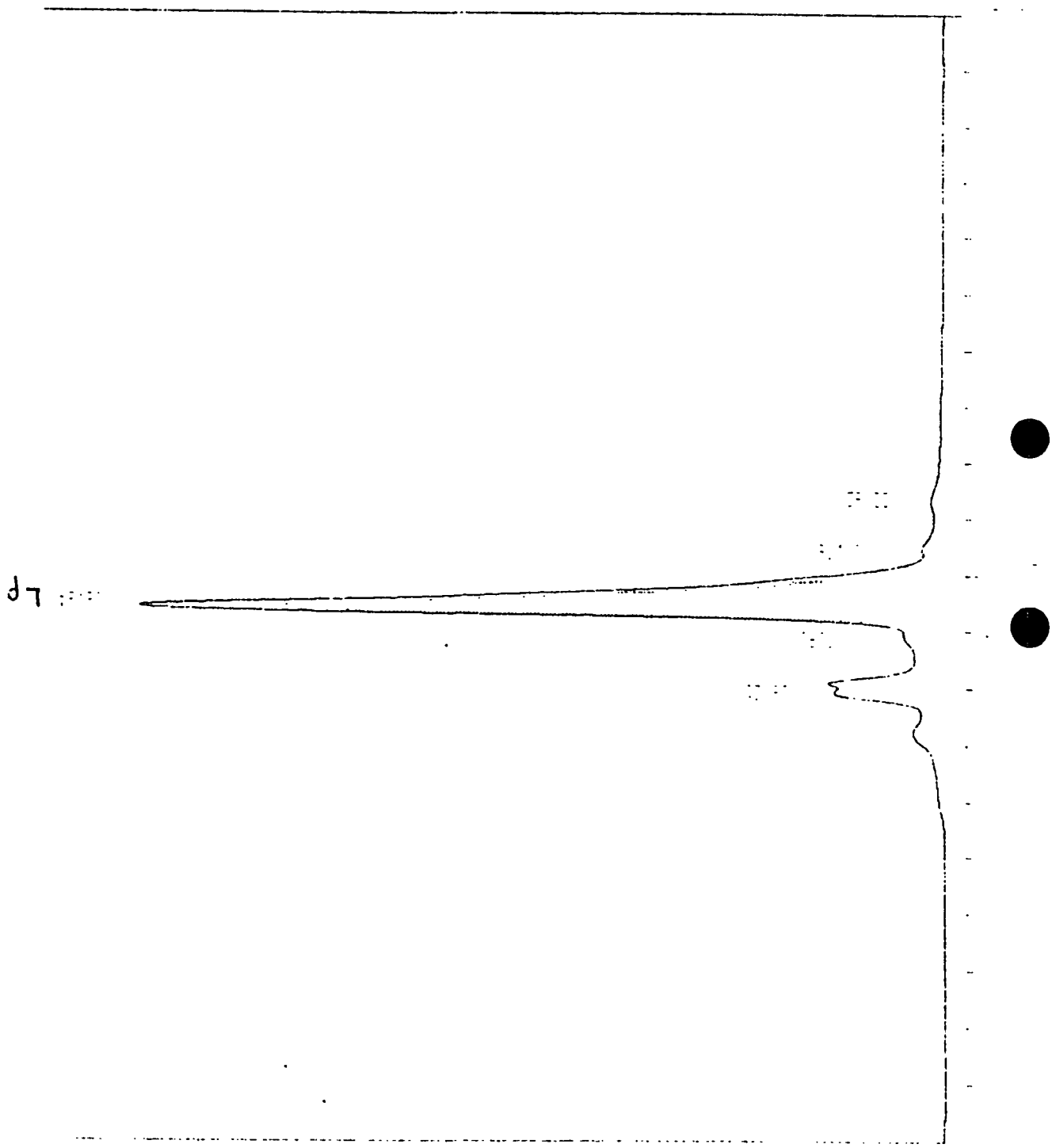


Figure 12
SP 5PW HPLC of Lactoperoxidase (BR 70 Fractions)

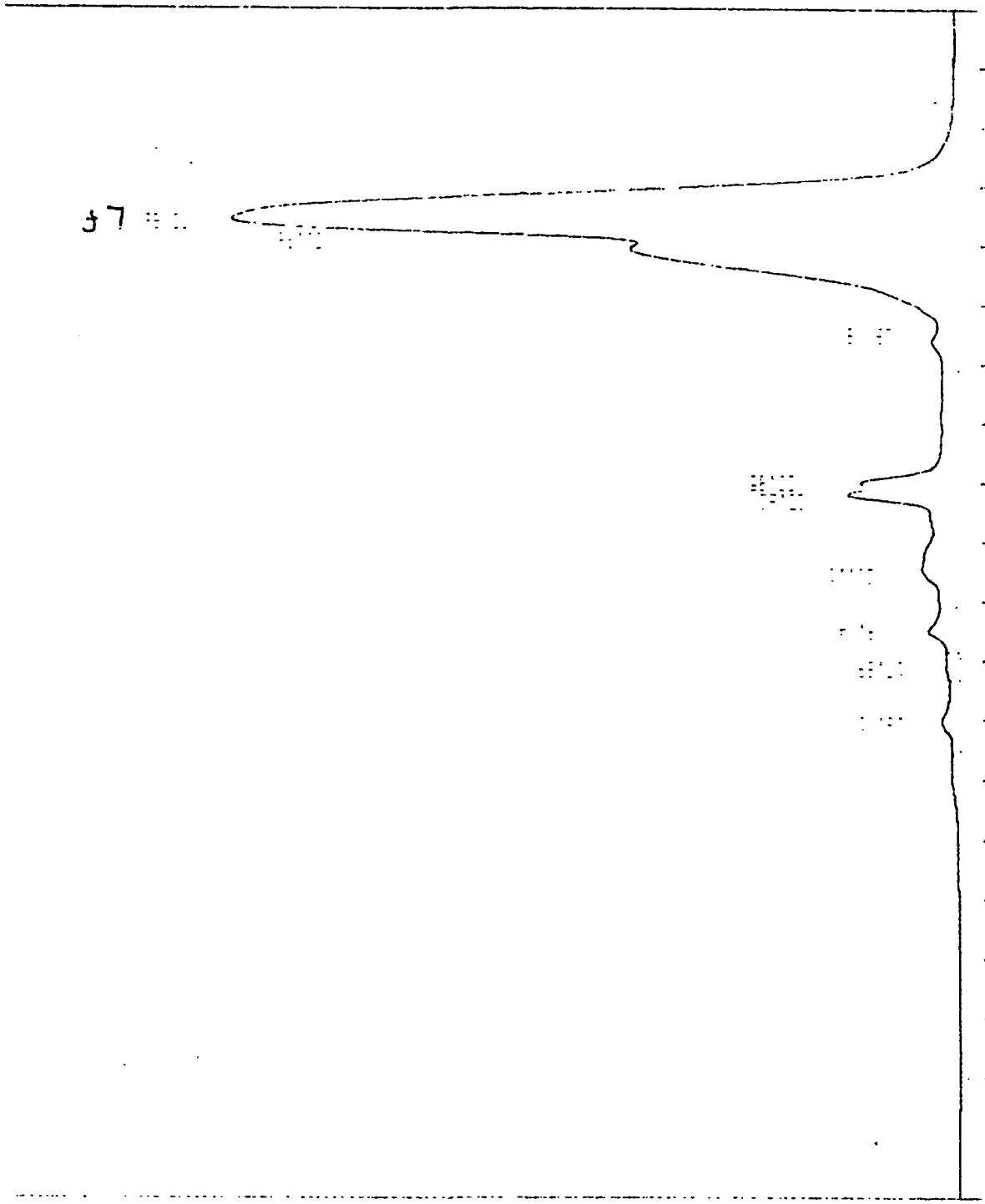
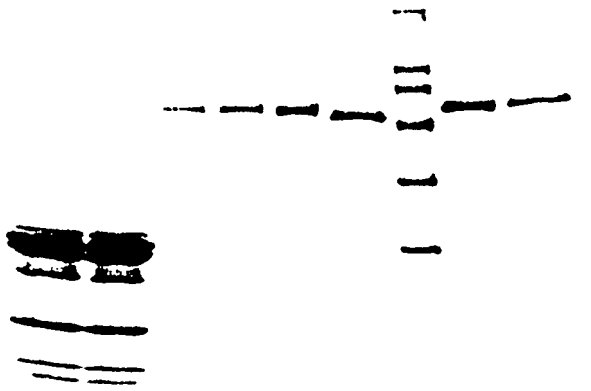
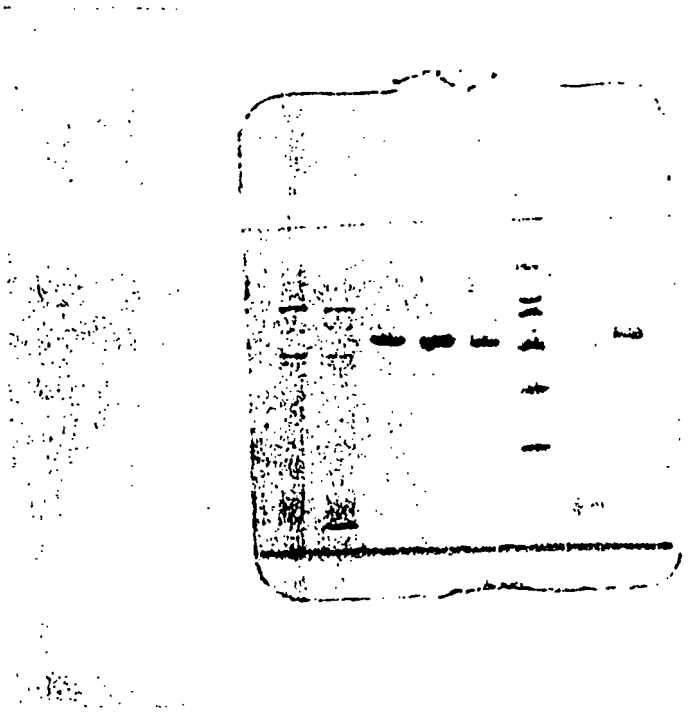


Figure 13
SP 5PW HPLC of Lactoferrin (BR 70 Fractions)



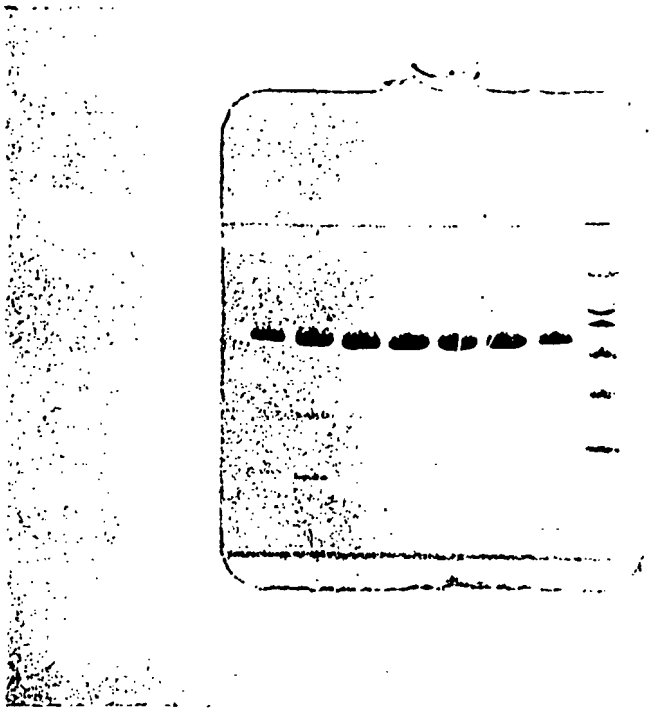
1 2 3 4 5 6 7 8 9

Figure 14 Fractions at several stages of the purification: lanes 1, whole milk = BR 70 load; 2, whole milk after BR 70 exposure; 3, Lp peak fractions pooled 6-13 from BR 70 column run 13 (Fig. 4); 4, Lf peak fractions pooled 21-32 from BR 70 column run 13 (Fig. 4); 5, Lp peak fractions pooled 14 and 15 (19 and 20 min) from SP 5PW HPLC column run 26 (Fig. 11); 6, Bovine Lp standard protein (Sigma Chemical Co.) 77.5 kDa; 7, SDS 6H molecular weight markers top to bottom: rabbit muscle myosin 205 kDa, E. coli β-galactosidase 116 kDa, rabbit muscle phosphorylase b 97.4 kDa, bovine albumin 66 kDa, egg albumin 45 kDa, and bovine carbonic anhydrase 29 kDa; 8, Lf peak fractions pooled 36-38 (32-34 min) from SP 5PW HPLC column run 27 (Fig. 12); 9, Bovine Lf standard protein (Sigma Chemical Co.) 83.1 kDa.



1 2 3 4 5 6 7 8

Figure 15 Fractions from the SP 5PW HPLC purification of Lp: lanes 1, 16 min; 2, 17 min; 3, 19 min; 4, 20 min; 5, Bovine Lp standard protein (Sigma Chemical Co.) 77.5 kDa; 6, SDS 6H molecular weight markers top to bottom: rabbit muscle myosin 205 kDa, E. coli β-galactosidase 116 kDa, rabbit muscle phosphorylase b 97.4 kDa, bovine albumin 66 kDa, egg albumin 45 kDa, and bovine carbonic anhydrase 29 kDa; 7, 18 min; 8, 21 min.

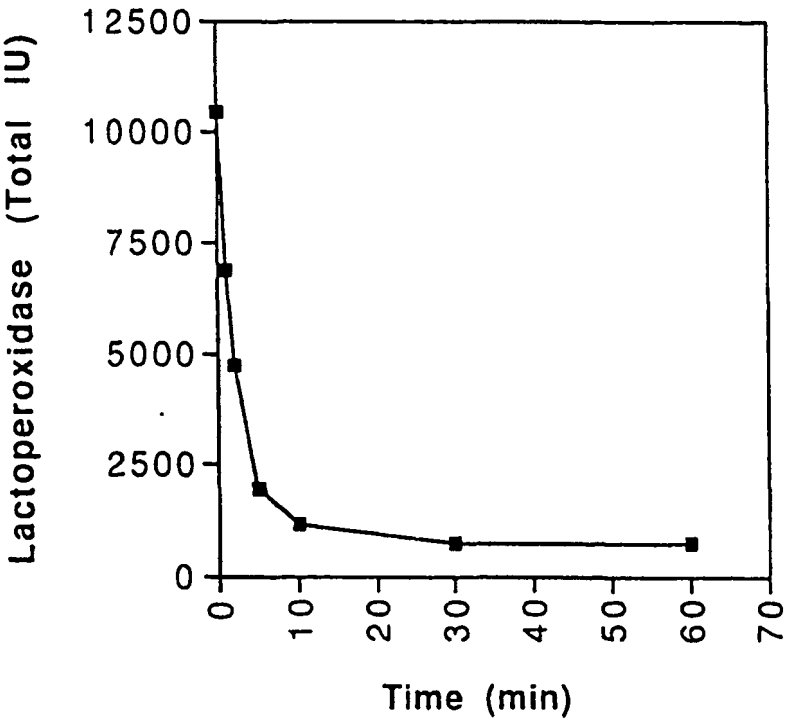


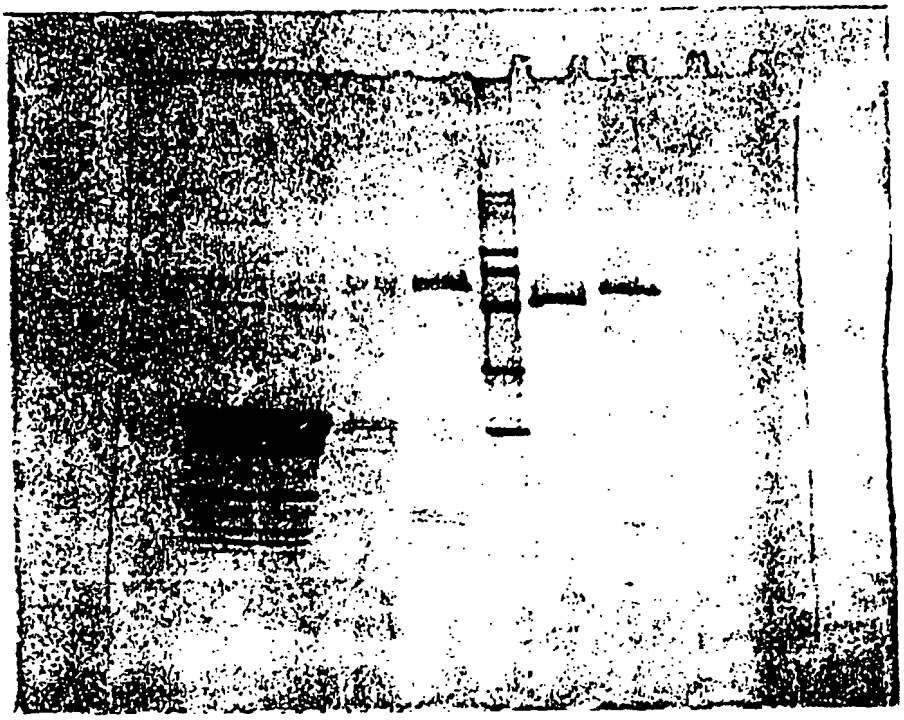
1 2 3 4 5 6 7 8

Figure 16 Fractions from the SP 5PW HPLC purification of Lf: lanes 1, 29 min; 2, 30 min; 3, 31 min; 4, 32 min; 5, 33 min; 6, 34 min; 7, Bovine Lf standard protein (Sigma Chemical Co.) 83.1 kDa; 8, SDS 6H molecular weight markers top to bottom: rabbit muscle myosin 205 kDa, E. coli β-galactosidase 116 kDa, rabbit muscle phosphorylase b 97.4 kDa, bovine albumin 66 kDa, egg albumin 45 kDa, and bovine carbonic anhydrase 29 kDa.

Figure 17

BRIEF Cartridge Capture of Lactoperoxidase from Whole Milk





1 2 3 4 5 6 7

Figure 18 Fractions at several stages of the purification: lanes 1, whole milk = BRIEF cartridge load; 2, whole milk after 30 min BRIEF cartridge exposure; 3, Lp 0.25 M NaCl eluted fraction; 4, Lf 0.8 M eluted fraction; 5, SDS 6H molecular weight markers top to bottom: rabbit muscle myosin 205 kDa, E. coli β-galactosidase 116 kDa, rabbit muscle phosphorylase b 97.4 kDa, bovine albumin 66 kDa, egg albumin 45 kDa, and bovine carbonic anhydrase 29 kDa; 6, Bovine Lp standard protein (Sigma Chemical Co.) 77.5 kDa; 7, Bovine Lf standard protein (Sigma Chemical Co.) 83.1 kDa.

END